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THE ROLE OF SUPEROXIDE RADICALS IN THE OXYGEN ENHANCEMENT OF RA--ETC(U)

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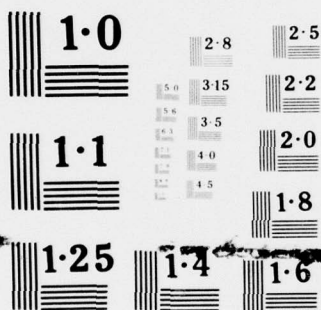
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## REPORT DOCUMENTATION PAGE

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1. REPORT NUMBER DAHC04 74 G 0194; DAAG29 76 G 0217	2. JOINT ACCESSION NO. (12) ARO	3. RECIPIENT'S CATALOG NUMBER (12) 12410.1-L
4. TITLE (and Subtitle) The Role of Superoxide Radicals in the Oxygen Enhancement of Radiation Damage.		5. TYPE OF REPORT & PERIOD COVERED Final July 1, 1974 - June 29, 1977
7. AUTHOR(s) (10) Irwin/Fridovich		6. PERFORMING ORG. REPORT NUMBER
8. CONTRACT OR GRANT NUMBER(s) (15) DAHC04-74-G-0194, DAAG29-76-G-0217		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS (11) 29 Jun 77
9. PERFORMING ORGANIZATION NAME AND ADDRESS Duke University Medical Center Durham, N. C. 27710		12. REPORT DATE 6/29/77
11. CONTROLLING OFFICE NAME AND ADDRESS U. S. Army Research Office Post Office Box 12211 Research Triangle Park, NC 27709		13. NUMBER OF PAGES (12) 14p.
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office) Submitted 9/1/77		15. SECURITY CLASS. (of this report) Unclassified
15. DISTRIBUTION STATEMENT (of this Report) Approved for public release; distribution unlimited.		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE NA
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report) NA (9) Final rept. 1 Jul 74-29 Jun 77,		DDC RECEIVED NOV 21 1977 B
18. SUPPLEMENTARY NOTES The findings in this report are not to be construed as an official Department of the Army position, unless so designated by other authorized documents.		
19. KEY WORDS (Continue on reverse side if necessary and identify by block number) superoxide radical superoxide dismutase oxygen enhancement ratio		
20. ABSTRACT (Continue on reverse side if necessary and identify by block number) We have found that the oxygen enhancement of the lethality of X-ray irradiation of dilute suspensions of <i>Escherichia coli</i> is strikingly diminished by superoxide dismutase or by catalase, added to the suspending medium. This implicates both $O_2^-$ and $H_2O_2$ as agents of the O.E.R. Since $O_2^-$ and $H_2O_2$ have previously been seen to lead to the production of $OH\cdot$ in biochemical systems,		

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~~we tested~~ <sup>are tested</sup> the effects of scavengers of such radicals. As expected, mannitol or histidine also decreased the O.E.R.

Since catalase and superoxide dismutase could be shown not to move from the medium into the cells, the protections caused by these enzymes were due to actions in the suspending medium. Hence  $O_2^{\cdot -}$  and  $H_2O_2$  generated in the medium are intermediates in the O.E.R.

We have actually worked on a number of related problems during the tenure of this grant and a bibliography of the papers published by us, during this time is appended.

The abstracts from selected papers will give a good idea of the work which we have accomplished under the Army grant.

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## Isolation and Characterization of a Manganese-containing Superoxide Dismutase from Yeast\*

(Received for publication, January 10, 1975)

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The cyanide-insensitive superoxide dismutase of yeast has been shown to be localized in the mitochondrial matrix. This enzyme has been isolated in good yield from bakers' yeast. Its molecular weight is 96,000. It is a tetramer, being composed of four subunits of equal size. Exposure to sodium dodecyl sulfate at 100° caused dissociation into dimers, while similar treatment but in the presence of 2-mercaptoethanol caused complete dissociation into monomers. This enzyme contains 1 atom of manganese per subunit and its absorption in the visible suggests Mn(III) in the resting enzyme. Ascorbate caused partial bleaching, presumably by reduction to Mn(II). The amino acid composition was determined. This enzyme has activity comparable to that of other previously reported superoxide dismutases and like the chicken mitochondrial and the bacterial enzymes, its rate of reaction with O<sub>2</sub> falls as the pH is raised above 7.8. Crystals of high quality were easily prepared.

Superoxide dismutases, which are unique among enzymes in that their substrate is a free radical, have been isolated and studied from a variety of respiring organisms. They serve the purpose of muting the toxicity of molecular oxygen by catalytically scavenging the superoxide-free radical, by way of the reaction:  $O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$  (1). Superoxide dismutases bearing copper and zinc, or manganese, or iron have been described. These have been found in specific places. Thus, the copper and zinc enzymes are characteristic of the cytosols of eukaryotic cells (1); the iron enzymes have been found in bacteria (2-4) and in blue-green algae (5, 6) and the manganese-containing enzymes have been seen in bacteria (7, 8) and in mitochondria (9). Studies of amino acid sequences have demonstrated that the manganese and the iron-containing superoxide dismutases from *Escherichia coli* are closely related to the manganese-containing enzyme from chicken liver mitochondria, whereas the copper and zinc enzyme from bovine erythrocytes was unrelated to any of the others (10). This result has obvious implications concerning the origin of mitochondria (11). Thus far, only one mitochondrial superoxide dismutase has been isolated and that was obtained from chicken liver (9). Further study of the evolutionary implications of the close relationship between the prokaryotic and the mitochondrial enzymes requires that additional mitochondrial superoxide dismutases be studied. A copper- and zinc-containing superoxide dismutase has been isolated from yeast (12) and was found to be similar to the corresponding enzymes found in the cytosols of other eukaryotic cells. We here report the isolation of a manganese-containing superoxide dismutase from *Saccharomyces cerevisiae*, which appears to be localized

in the mitochondria of this organism and whose properties indicate a close relationship to the chicken liver mitochondrial enzyme.

### MATERIALS AND METHODS

Cytochrome c, type III, and nitroblue tetrazolium were obtained from the Sigma Chemical Co., while carboxymethyl cellulose and diethylamino ethyl cellulose were products of the Reeve Angel Co. The ion exchange celluloses were alternately washed with 0.5 M NaOH, water, and 0.5 M HCl. Water was used to wash away the acid or alkali and the DEAE-cellulose, in the alkaline form, was finally equilibrated with water while the CM-cellulose, in the acid form, was equilibrated with 0.01 M sodium acetate at pH 5.2. Air-dried bakers' yeast was a product of the Fleischman Co. Calcium phosphate gel was prepared according to Keilin and Hartree (13). Xanthine oxidase was prepared from cream according to Brady (14).

Superoxide dismutase was assayed either in terms of its ability to inhibit the reduction of cytochrome c by milk xanthine oxidase plus xanthine (15) or the reduction of nitroblue tetrazolium by a photochemical flux of O<sub>2</sub> (16). The latter assays were performed at 25° by mixing 1.0 ml of 0.05 M potassium phosphate buffer at pH 7.8 containing 0.1 mM EDTA, 0.1 ml of 1 mM nitroblue tetrazolium, 0.15 ml of 0.1 M methionine, 30  $\mu$ l of 0.16 mM riboflavin, and enzyme sample plus water to a final volume of 3.0 ml and by then exposing this mixture to fluorescent light, at such intensity that the absorbance at 550 nm in the absence of enzyme increased at a rate of 0.05/min. One unit of superoxide dismutase was defined as that amount which caused 50% inhibition.

The cytochrome c reduction assay for superoxide dismutase activity was 2.5-fold less sensitive than was the photochemical nitroblue tetrazolium reduction assay. Thus, one unit of activity in the former assay equaled 2.5 units in the latter assay. Electrophoresis on polyacrylamide gels was performed according to Davis (17) and superoxide dismutase activity was localized on these gels as previously described (16). The cyanide-sensitive copper- and zinc-containing enzyme was differentiated from the cyanide-insensitive manganese-containing enzyme by performing these activity stains in the presence and in the absence of millimolar cyanide (9).

The molecular weight of the enzyme was determined by the method

\* This work was supported in full by Research Grants GM-10287 from the National Institutes of Health and RDRP-IP-12410-L from the United States Army Ordinance Research Office.



### A Convenient Calibration of the Clark Oxygen Electrode<sup>1</sup>

The use of plastic-covered platinum electrodes (1) for the polarographic estimation of dissolved oxygen has become routine in studies of oxygen-consuming reactions. The problem of calibrating these electrodes has been solved in several ways. Admittedly, the simplest of these is to use the known solubility of oxygen, in conjunction with its partial pressure in air, to calculate the concentration of  $O_2$  in air-equilibrated solutions. This calculated concentration can then be set to full scale on the microammeter being used to measure the current due to reduction of oxygen at the electrode. This method lacks precision because it is sensitive to the ionic strength and the temperature of the solution during calibration and to the partial pressure of oxygen in the environment, and it does not allow for calibration at a sensitivity greater than  $2.4 \times 10^{-4} M O_2 = \text{full scale}$ . An early chemical method of calibration used known amounts of NADH, in the presence of submitochondrial particles, to consume known amounts of oxygen (2). The necessity of preparing submitochondrial particles for use in this method was eliminated by Robinson and Cooper (3), who utilized the catalytic properties of phenazinium methylsulfate to oxidize known amounts of NADH. Catalase was also used in this method to convert  $H_2O_2$  to  $H_2O$ , and thus to eliminate imprecision due to the variable yields of these two reduction products of  $O_2$ . Still another method of calibration utilized xanthine oxidase plus xanthine to consume known amounts of  $O_2$  (4).

A method which entirely avoids the use of enzymes and which is based only upon readily available and inexpensive compounds is desirable. We now describe such a method which is based only upon phenylhydrazine and ferricyanide. Phenylhydrazine is quite stable in acid solutions, but in neutral to alkaline solutions it slowly autoxidizes by way of an exceedingly complex autocatalytic, free-radical chain reaction (5). Phenylhydrazine is rapidly oxidized to phenyldiimide by ferricyanide (6), and phenyldiimide reacts very rapidly with  $O_2$  (7). The method described below exploits these properties of phenylhydrazine in the presence of ferricyanide.

#### MATERIALS AND METHODS

Phenylhydrazine hydrochloride was obtained from the Sigma Chemical Company, and potassium ferricyanide was obtained from Matheson.

<sup>1</sup> This work was supported in full by research Grants GM-10287 and HL-17603 from the National Institutes of Health, Bethesda, Maryland, and RDRP-IP-12410-L from U.S. Army Research Ordinance Office, Durham, North Carolina.

## The Oxidation of Phenylhydrazine: Superoxide and Mechanism<sup>†</sup>

Hara P. Misra and Irwin Fridovich\*

**ABSTRACT:** The oxidation of phenylhydrazine in buffered aqueous solutions is a complex process involving several intermediates. It can be initiated by metal cations, such as  $\text{Cu}^{2+}$ , in which case EDTA acts as an inhibitor. It can also be initiated by oxyhemoglobin, in which case chelating agents do not interfere. Superoxide radical is both a product of this reaction and a chain propagator. The formation of  $\text{O}_2^-$  could be demonstrated in terms of a reduction of nitroblue tetrazolium, which was prevented by superoxide dismutase. The importance of  $\text{O}_2^-$  in carrying the reaction chains was shown by the inhibition of phenylhydrazine oxidation by superoxide dismutase. Hydrogen peroxide accumulated during the reaction and could be detected with catalase. The progress of this oxidation could be monitored in terms of oxygen consumption and by following increases in absorbance at 280 or 320 nm. The oxidation was markedly autocatalytic and superoxide dismutase had the effect of extending the lag period. The absorbance at 280 nm was due

to an intermediate which first accumulated and was then consumed. This intermediate appears to be benzenediazonium ion. The absorbance at 320 nm was due to a stable product, which was not identified. The time course of oxygen consumption paralleled the increase in absorbance at 320 nm and lagged behind the changes at 280 nm. Exogenous benzenediazonium ion accelerated the oxidation of phenylhydrazine and eliminated the lag phase. Benzenediazonium ion must therefore react with phenylhydrazine to produce a very reactive intermediate, possibly phenyldiazene. A mechanism was proposed which is consistent with the data. The intermediates and products of the oxidation of phenylhydrazine include superoxide radical, hydrogen peroxide, phenylhydrazyl radical, phenyldiazene, and benzenediazonium ion. This is a minimal list; others remain to be detected and identified. It appears likely that the diverse biological effects of phenylhydrazine are largely due to the reactivities of these intermediates and products.

Phenylhydrazine has long been known as a hemolytic agent (Warburg et al., 1931; Lemberg and Legge, 1942; Beaven and White, 1954) and has been shown to increase the production of  $\text{H}_2\text{O}_2$  within intact human erythrocytes (Cohen and Hochstein, 1964). Several of the effects of phenylhydrazine are oxygen-dependent. These include its inactivation of papain (Allison and Swain, 1973), amine oxidase (Patek and Hellerman, 1974), lactoperoxidase and triosephosphate dehydrogenase (Allison et al., 1973), horse radish peroxidase and thyroid peroxidase (Hidaka and Udenfriend, 1970; Hidaka et al., 1970), its inhibition of the respiration of mitochondria and of submitochondrial particles (Asami, 1968), and its desensitization of grasshopper muscle (McDonald, 1972). Aside from the involvement of phenyldiazene as an intermediate (Cauquis and Genies, 1968; Itano, 1970), the catalytic effect of  $\text{Cu}^{2+}$  (Ebersson and Persson, 1962; Audrieth and Ogg, 1951) and the formation of  $\text{H}_2\text{O}_2$  (Cohen and Hochstein, 1964; Audrieth and Ogg, 1951), very little is known about the air oxidation of phenylhydrazine in buffered aqueous solutions.

The reduction of molecular oxygen to  $\text{H}_2\text{O}_2$  frequently occurs in univalent steps with  $\text{O}_2^-$  as an intermediate. This has been shown to be the case during the reduction of oxygen by epinephrine (Misra and Fridovich, 1972b), pyrogallol (Marklund and Marklund, 1974), oxyhemoglobin (Misra and Fridovich, 1972c), reduced ferredoxins (Misra

and Fridovich, 1971) and leucoflavins, hydroquinones, and thiols (Misra and Fridovich, 1972a; Misra, 1974). It therefore seemed reasonable that this might also be true of the oxidation of phenylhydrazine, in which case  $\text{O}_2^-$  and other radical intermediates would have to be considered as possible agents of the biological effects of this compound. The ability of  $\text{O}_2^-$  to reduce nitroblue tetrazolium (Beauchamp and Fridovich, 1971) and the availability of superoxide dismutases, which catalytically scavenge  $\text{O}_2^-$  (Fridovich, 1974), provided the means for probing the oxidation of phenylhydrazine. The report which follows describes studies of the mechanism of oxidation of phenylhydrazine.  $\text{O}_2^-$ , phenyldiazene, and benzenediazonium ion appear to be intermediates and a tentative mechanism is proposed. While this work was in progress,  $\text{O}_2^-$  was reported to be a product of the oxidation of phenylhydrazine by oxyhemoglobin (Goldberg and Stern, 1975).

### Materials and Methods

**Hemoglobin.** Heparinized human blood was centrifuged and the erythrocytes were washed three times with isotonic saline and were then lysed by admixture of an equal volume of water. Cell stroma were removed by centrifugation at 100 000g for 1 h. A small amount of  $\text{Na}_2\text{S}_2\text{O}_4$  (~1.0 mg/ml) was added to ensure complete reduction of the hemoglobin, which was then separated from smaller molecules by gel exclusion chromatography of 12 ml of the lysate on a 4 × 80 cm column of Sephadex G-75, which had been equilibrated and was eluted with nitrogen-purged 0.05 M potassium phosphate-0.10 M KCl at pH 7.4. The hemoglobin-containing eluate was found to be free of superoxide dismutase. It was converted to oxyhemoglobin by oxygenation and to methemoglobin by treatment with potassium ferricyanide

<sup>†</sup> From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710. Received June 9, 1975. This work was supported in part by Research Grants GM-10287 and HL-17603 from the National Institutes of Health, Bethesda, Md., and RDRP-IP-12410-L from the U.S. Army Ordinance Research, Durham, N.C.

## The Mechanism of the Activity-Dependent Luminescence of Xanthine Oxidase<sup>1</sup>

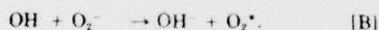
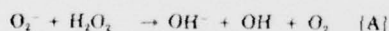
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Received June 30, 1975

The weak luminescence that accompanies the aerobic xanthine oxidase reaction is inhibited by superoxide dismutase, by catalase, and by scavengers of hydroxyl radicals. It is also entirely dependent upon the presence of carbonate. It thus appears that the  $O_2^-$  and  $H_2O_2$  produced during the aerobic action of xanthine oxidase interact to generate  $OH^-$  which, in turn, reacts with carbonate to yield the carbonate radical ( $CO_3^{\cdot-}$ ). The species that is directly responsible for light emission appears to be produced by a dimerization of carbonate radicals, since the light intensity was a function of the square of the carbonate concentration. The data provide no reason to suppose that the light-emitting species is singlet oxygen.

Xanthine oxidase causes a weak luminescence while catalyzing the aerobic oxidation of its substrates (1, 2). Arneson (3) observed that this luminescence was inhibited by catalase or by superoxide dismutase and deduced that both  $O_2^-$  and  $H_2O_2$  were essential intermediates in the light-producing process. Since it had previously been suggested (4-6) that singlet oxygen could emit in the visible by the formation of dimers, followed by pooling and simultaneous emission of excitation energy, Arneson (3) concluded that the emitting species in the xanthine oxidase reaction was singlet oxygen produced by the following reactions.



More recently Stauff and co-workers (7) have shown that the luminescence accompanying the oxidation of  $H_2O_2$  by periodate, previously attributed to singlet oxygen, is actually dependent upon carbonate. They suggested that the actual emitting species was produced by a reaction involving carbonate radicals. We have reinvesti-

gated the production of light during the xanthine oxidase reaction and have demonstrated that carbonate is essential for this luminescence. The actions of catalase, superoxide dismutase and scavengers of hydroxyl radical were also reinvestigated, and the results lead to a reaction scheme that accounts for the properties of this system.

### MATERIALS AND METHODS

Xanthine oxidase was prepared from cream by a procedure that avoids proteolysis (8). Catalase was from Sigma Chemical Company and was freed of contaminating superoxide dismutase (9) by repeated washing on an XM100A Diaflo ultrafiltration membrane obtained from the Amicon Corporation. Catalase activity was assayed at 25°C by the method of Beers and Sizer (10). The manganese-containing superoxide dismutase was prepared from *Escherichia coli* as previously described (11). The acetaldehyde used was freshly distilled daily. All other materials used were reagent grade. Luminescence measurements were made with either a Nuclear Chicago Mark I liquid scintillation counter or a Packard Tri-Carb scintillation spectrometer, Model 3003. In either case the coincidence circuit was turned off and the signal from a control vial was subtracted from the signal given by the luminescent reaction.

### RESULTS

#### The Dependence upon Carbonate

When  $4 \times 10^{-2}$  M xanthine oxidase catalyzed the oxidation of 10 mM acetaldehyde

<sup>1</sup> This work was supported in full by research grants, No. GM-10287 from the National Institutes of Health, Bethesda, Md., and No. RDRP-IP-12410-L from the United States Army Research Office, Durham, N. C.



## Superoxide, Hydrogen Peroxide, and Singlet Oxygen in Lipid Peroxidation by a Xanthine Oxidase System\*

(Received for publication, July 21, 1975)

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1. Xanthine oxidase acting aerobically upon acetaldehyde was found to cause the peroxidation of linolenate. This was demonstrated by increased absorbance at 233 nm due to diene conjugation and by the detection of a lipid peroxide spot on thin layer chromatograms.

2. Superoxide dismutase inhibited this lipid peroxidation, as did catalase, thus indicating that both  $O_2^-$  and  $H_2O_2$  were essential intermediates. Scavengers of singlet oxygen also inhibited the peroxidation of linolenate, whereas scavengers of hydroxyl radical did not. These effects, which were observed in the absence of iron salts, led to the proposal that  $O_2^-$  and  $H_2O_2$  can directly give rise to singlet oxygen, as follows:  $O_2^- + H_2O_2 \rightarrow OH^- + OH \cdot + O_2^*$ .

3. This proposal was further supported through the use of 2,5-dimethylfuran, as an indicating scavenger of singlet oxygen. Thus, when this compound was exposed to a known source of singlet oxygen, it gave a product which was detectable by thin layer chromatography. This product was also observed when 2,5-dimethylfuran was exposed to the xanthine oxidase system, in which case its accumulation was prevented by superoxide dismutase or by catalase, but not by scavengers of hydroxyl radical.

There recently has been a surge of interest in the peroxidation of lipids. This interest follows upon the early observations by Hochstein *et al.* (1-2a) of an enzymatic, NADPH-dependent oxidation of microsomal lipids, and it derives from the obvious importance of unsaturated lipids for the structural and functional integrity of biological membranes. This literature is ample but its impact is unclear. Iron salts and chelating agents, such as ADP or EDTA, have been used routinely by most workers because they have appeared to augment the rate of lipid peroxidation. Pederson and Aust (3, 4) noted that xanthine oxidase, while acting aerobically upon xanthine, could cause lipid peroxidation. Since scavengers of  $O_2^-$  and of singlet oxygen inhibited, they concluded that  $O_2^-$ , generated by xanthine oxidase, gave rise in turn to singlet oxygen which was then the immediate cause of the lipid peroxidation. The reactivity of singlet oxygen with unsaturated lipids (5-8) makes this a very reasonable supposition. Superoxide dismutase has been used repeatedly to expose the involvement of  $O_2^-$  in the chain of events leading to lipid peroxidation by the xanthine oxidase system (3, 4, 9, 10), but the presence of chelated iron salts adds a complicating factor which hinders interpretation of these data. In one study (11), the xanthine oxidase system was seen to mount an oxidative attack upon the lipids of lysosomal membranes. In this case, superoxide dismutase augmented lysis rather than inhibiting it, and  $OH \cdot$  was

implicated as the causative agent. Here too, iron salts were present to cloud the issues.

It seemed necessary to explore the co-oxidation of unsaturated lipids by the xanthine oxidase system, under simple and well defined conditions, so that mechanisms might be discerned more readily. The peroxidation of linolenate has been studied in this way. The results of these studies and the mechanism they suggest form the basis of this report.

### MATERIALS AND METHODS

Thiobarbituric acid, mannitol,  $\beta$ -carotene, L-ascorbic acid, catalase, bovine serum albumin, and hemoglobin were obtained from the Sigma Chemical Co. The catalase was occasionally dialyzed, to free it of a thymol preservative, and it was also freed of a minor contamination with superoxide dismutase by repeated washing over an XM-100 A Diaflo ultrafiltration membrane from the Amicon Corp., without any effect on the results. Starch (Soluble, Lintner) and *t*-butyl alcohol were purchased from the Fisher Scientific Co. EDTA and trichloroacetic acid were from Matheson, Coleman and Bell, while potassium iodide and ferric chloride were from Baker and Adamson. Linolenic acid was obtained from Mann, and was stored *in vacuo* at  $-20^\circ$ . Potassium phosphates were from J. T. Baker and, unless otherwise specified, buffers made from these salts were passed through a Chelex 100 column, to remove trace metals. The Chelex 100 was from Bio-Rad Laboratories, Inc. Dimethoxyethane, from Eastman Chemical Co., was stored over metallic sodium and was passed through a column of alumina, to remove peroxides, on the day of use. 2,5-Dimethylfuran, 2,5-diphenylfuran, and 1,4-diazabicyclo[2.2.2]octane were purchased from the Aldrich Chemical Co. The dimethylfuran was distilled, and the fraction collected between  $83-85^\circ$  was used where indicated. In other cases, it was used as supplied. Diphenylfuran was recrystallized from 95% ethanol, while diazabicyclooctane was recrystallized from acetone. Superoxide dismutase from bovine erythrocytes was purified by Truett Laboratories as previously described (12). It was freed of

\* This work was supported in full by Research Grants GM-10287 and HL-17603 from the National Institutes of Health, Bethesda, Maryland and RDRP-I-P-12140-L from the United States Army Research Office, Durham, North Carolina 27709.

# The Interaction of Bovine Erythrocyte Superoxide Dismutase with Hydrogen Peroxide: Chemiluminescence and Peroxidation<sup>†</sup>

Ellen K. Hodgson and Irwin Fridovich\*

**ABSTRACT:** Reaction of bovine erythrocyte superoxide dismutase with  $H_2O_2$  was accompanied by a luminescence whose intensity was a function of the concentration of  $H_2O_2$  and whose duration was coincident with the inactivation of the enzyme by this reagent. Oxygen, which protected against inactivation, also diminished the luminescence. Several other compounds which prevented the inactivation by  $H_2O_2$  also modified the luminescence. Thus urate, formate, and triethylamine inhibited luminescence whereas imidazole and xanthine augmented it. These seemingly contrary effects can be explained by assuming that the compounds which protected the enzyme were peroxidized in competition with the sensitive group on the enzyme. The luminescence arises because that group on the enzyme was oxidized to a product in an electronically excited state, which could

return to the ground state by emitting light. Imidazole and xanthine gave electronically excited products whose quantum efficiency was greater than that of the group on the enzyme, whereas urate, formate, and triethylamine gave products with much lower luminescent efficiencies. This superoxide dismutase could catalyze the peroxidation of a wide range of compounds, including ferrocytochrome *c*, luminol, diphenylisobenzofuran, dianisidine, and linoleic acid. In control experiments, boiled enzyme was inactive. This peroxidative activity can lead to unexpected effects when superoxide dismutase is added to  $H_2O_2$ -producing systems, as a probe for the involvement of  $O_2^-$ . Several examples from the literature are cited to illustrate the misinterpretations which this previously unrecognized peroxidative activity can generate.

The preceding paper (Hodgson and Fridovich, 1975) describes the inactivation of the copper- and zinc-containing superoxide dismutase by  $H_2O_2$ . A mechanism was proposed in which  $H_2O_2$  first reduces the  $Cu^{2+}$  and then reacts with the  $Cu^+$ , so generated, to give a potent oxidant, which remains bound to the metal. This bound oxidant, in turn, attacks an adjacent histidine residue and so destroys the integrity of the catalytic site. In the course of these studies we observed a chemiluminescence during the reaction of  $H_2O_2$  with the enzyme and noted additionally that superoxide dismutase can act as a peroxidase. Since superoxide dismutase is often used as a test for  $O_2^-$  in oxidative and in chemiluminescent reactions and since  $H_2O_2$  is often a product of such reactions, this peroxidative action of superoxide dismutase can, if not appreciated, lead to misinterpretation of the observations. The chemiluminescence and the peroxidations which accompany the interaction of superoxide dismutase with  $H_2O_2$  were therefore studied both to gain understanding of their mechanisms and to expose the dangers of

uncritically applying superoxide dismutase as a test for  $O_2^-$  in peroxide-generating systems.

## Materials and Methods

The manganese-containing superoxide dismutase was prepared from *Escherichia coli* as previously described (Keele et al., 1970). Luminescent intensity was measured with the photometer described by Mitchell and Hastings (1971) which was calibrated with the stable standard light source described by Hastings and Weber (1963). Some of the measurements were made with a Nuclear Chicago Mark I liquid scintillation counter with the coincidence circuit inactivated. Rates of change of absorbance were recorded with a Gilford Model 2000. The absorption spectrum of cytochrome *c* was recorded with an Aminco DW-2 whereas the spectrum of linoleic acids was taken with a Cary Model 15. The bleaching of diphenylisobenzofuran was followed at 410 nm (Merkel et al., 1972). The peroxidation of dianisidine was followed at 460 nm (Fridovich, 1963). All other materials and procedures were exactly as described in the preceding paper (Hodgson and Fridovich, 1975).

## Results

### *Chemiluminescence. Admixture of superoxide dismutase*

<sup>†</sup> From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710. Received June 2, 1975. This work was supported in full by Research Grants GM-10287 from the National Institutes of Health, Bethesda, Maryland, and RDRP-IP-12410-L from the U.S. Army Ordinance Research Office, Durham, North Carolina.

## The Interaction of Bovine Erythrocyte Superoxide Dismutase with Hydrogen Peroxide: Inactivation of the Enzyme<sup>†</sup>

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**ABSTRACT:** Bovine erythrocyte superoxide dismutase was slowly and irreversibly inactivated by hydrogen peroxide. The rate of this inactivation was directly dependent upon the concentrations of both  $\text{H}_2\text{O}_2$  and of enzyme, and its second-order rate constant at pH 10.0 and 25° was  $6.7 \text{ M}^{-1} \text{ sec}^{-1}$ . Inactivation was preceded by a bleaching due to rapid reduction of  $\text{Cu}^{2+}$  on the enzyme, and following this there was a gradual reappearance of a new absorption in the visible region, which was coincident with the loss of catalytic activity. Inactivation of the enzyme was pH-dependent and indicated an essential ionization whose  $\text{pK}_a$  was approximately 10.2. Replacement of  $\text{H}_2\text{O}$  by  $\text{D}_2\text{O}$  raised this  $\text{pK}_a$  but did not diminish the catalytic activity of superoxide dismutase, measured at pH 10.0. Several compounds, in-

cluding xanthine, urate, formate, and azide, protected the enzyme against inactivation by  $\text{H}_2\text{O}_2$ . Alcohols and benzoate, which scavenge hydroxyl radical, did not protect. Compounds with special affinity for singlet oxygen were similarly ineffective. The data were interpreted in terms of the reduction of the enzyme-bound  $\text{Cu}^{2+}$  to  $\text{Cu}^+$ , by  $\text{H}_2\text{O}_2$ , followed by a Fenton's type reaction of the  $\text{Cu}^+$  with additional  $\text{H}_2\text{O}_2$ . This would generate  $\text{Cu}^{2+}\text{-OH}\cdot$  or its ionized equivalent,  $\text{Cu}^{2+}\text{-O}\cdot^-$ , which could then oxidatively attack an adjacent histidine and thus inactivate the enzyme. Compounds which protected the enzyme could have done so by reacting with the bound oxidant, in competition with the adjacent histidine.

**S**uperoxide dismutases catalyze the reaction  $\text{O}_2^{\cdot-} + \text{O}_2^{\cdot-} + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$ . By thus scavenging  $\text{O}_2^{\cdot-}$  they serve to protect respiring cells against its deleterious reactivities.

The superoxide dismutase of bovine erythrocytes is characteristic of the corresponding enzymes found in the cytosols of eukaryotes and it is the most thoroughly studied of these enzymes (Fridovich, 1974, 1975). Its molecular weight is 32000 and it is made up of two identical subunits, each of which contains one  $\text{Cu}^{2+}$  and one  $\text{Zn}^{2+}$ . X-ray diffraction analysis has shown (Richardson et al., 1975) that the  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  are in close proximity, as was predicted by Fee and Gaber (1972). They are, in fact, joined by a common ligand, which is the imidazole ring of histidine-61. In addi-

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## Superoxide Dismutase and the Oxygen Enhancement of Radiation Lethality<sup>1</sup>

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Received April 12, 1976

*Escherichia coli* B were more susceptible to radiation lethality and showed a greater oxygen enhancement ratio when exposed in dilute suspension ( $1 \times 10^5$  cells/ml) than when exposed in dense suspensions ( $1 \times 10^9$  cells/ml). The oxygen enhancement, seen with dilute suspensions, was diminished by superoxide dismutase, catalase, mannitol, or histidine. Heat-denatured superoxide dismutase was without effect. The results are interpreted as indicating a role for  $O_2^-$  plus  $H_2O_2$  in the oxygen enhancement of radiation lethality, and a scheme is proposed which is consistent with the observations.

Exposure of *Escherichia coli* B to oxygen caused an increase in intracellular superoxide dismutase which correlated with increased resistance towards oxygen toxicity (1, 2). This induction of superoxide dismutase by molecular oxygen was used to explore the importance of  $O_2^-$  in the oxygen enhancement of radiation lethality (3). *E. coli* B which had been grown anaerobically and which consequently had a low level of superoxide dismutase were compared with cells which had grown aerobically and which had a higher level of this enzyme. Since superoxide dismutase is an effective scavenger of  $O_2^-$  and since the oxygen enhancement ratio (OER)<sup>2</sup> was the same in the aerobically grown as in the anaerobically grown cells, it was concluded (3) that  $O_2^-$  is not involved in the oxygen effect. It can be argued that these experiments tested the importance of intracellular  $O_2^-$  but not of extracellular  $O_2^-$  in the OER. Furthermore, superoxide dismutase, added to the suspending medium, has since been reported to protect fetal calf

myoblasts (4) and *Acholeplasma laidlawii* (5) against radiation lethality, and injected superoxide dismutase has similarly been reported to protect mice (6). It is clearly important to reinvestigate the role of  $O_2^-$  and of extracellular events in the oxygen effect on *E. coli* B. The results reported below indicate that  $O_2^-$  and  $H_2O_2$ , generated in the surrounding medium, are important intermediates of the OER in dilute suspensions of *E. coli* B.

### MATERIALS AND METHODS

Trypticase-soy broth was a product of the Bioquest Division of Becton, Dickinson. Yeast extract and Bacto-Agar were obtained from Difco. *Escherichia coli* B was obtained from the American Type Culture Collection, A T C C #23224. This organism was grown at 37°C in deep-still liquid medium composed of 30 g of trypticase-soy and 5 g of yeast extract per liter. A 0.1% inoculum was grown overnight and the resultant stationary-phase suspension was washed once and was then diluted to  $10^5$  cells/ml with 50 mM potassium phosphate, 2 mM  $MgSO_4$  at pH 7.0. These cells were then irradiated in a stirred shallow layer in plastic petri dishes under a stream of filtered air or of nitrogen. The nitrogen was passed sequentially through Fieser solution and a water scrubber before being passed over the cells. At least 15 min was allowed for equilibration with the gas stream before dosing with X rays, at room temperature. The X-ray source was a Picker X-Ray Corporation industrial unit, equipped with a Machlett Labs Type AEG-50 tube, operated at 171 V and

<sup>1</sup> This work was supported by Research Grants GM-10287 and HL-17603 from the National Institutes of Health, Bethesda, Md., and DAHC-0474-G-0194 from the United States Army Research Office, Research Triangle Park, N. C.

<sup>2</sup> Abbreviations used: OER, oxygen enhancement ratio; SOD, superoxide dismutase.



## Enzymatic Defenses Against the Toxicity of Oxygen and of Streptonigrin in *Escherichia coli*

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Received for publication 21 September 1976

Anaerobically grown *Escherichia coli* K-12 contain only one superoxide dismutase and that is the iron-containing isozyme found in the periplasmic space. Exposure to oxygen caused the induction of a manganese-containing superoxide dismutase and of another, previously undescribed, superoxide dismutase, as well as of catalase and peroxidase. These inductions differed in their responsiveness towards oxygen. Thus the very low levels of oxygen present in deep, static, aerobic cultures were enough for nearly maximal induction of the manganese-superoxide dismutase. In contrast, induction of the new superoxide dismutase, catalase, and peroxidase required the much higher levels of oxygen achieved in vigorously agitated aerobic cultures. Anaerobically grown cells showed a much greater oxygen enhancement of the lethality of streptonigrin than did aerobically grown cells, in accord with the proposal that streptonigrin can serve as an intracellular source of superoxide. Anaerobically grown cells in which enzyme inductions were prevented by puromycin were damaged by exposure to air. This damage was evidenced both as a decline in viable cell count and as structural abnormalities evident under an electron microscope.

The importance of superoxide dismutase (SOD) as a defense against the toxicity of oxygen has been supported by studies of the induction of this activity in *Escherichia coli* B. *E. coli* B has been reported to contain two superoxide dismutases, one of which contains manganese (12) and is found in the cell matrix (10), whereas the other contains iron (17) and is found in the periplasmic space (10). Exposure of *E. coli* B to oxygen caused an increase in manganese-containing SOD (MnSOD) which correlated with a gain in resistance towards hyperbaric oxygen (7, 10). Streptonigrin is more toxic in the presence of oxygen and seems to function as an intracellular source of  $O_2$  (16, J. R. White, T. O. Vaughan and W.-S. Yeh, Proc. Fed. Am. Soc. Exp. Biol. 30:1145, 1971). In accord with this view, increased levels of MnSOD in *E. coli* B also correlated with resistance towards streptonigrin (8). *E. coli* K-12 appeared to differ from *E. coli* B in its responses to oxygen. Thus, transfer from static aerobic cultures to forced aeration occasioned an induction of MnSOD in the case of *E. coli* B but not in *E. coli* K-12 (9). However, static liquid culture is not the same as completely anaerobic culture, and it appeared possible that the apparent difference between *E. coli* B and *E. coli* K-12 might be quantitative rather than qualitative. Thus *E. coli* K-12 might respond to such low concentrations of oxygen that its MnSOD was

almost fully induced by the low levels of  $O_2$  present in static liquid cultures. It thus appeared important to explore the responses of *E. coli* K-12 to oxygen. The induction of superoxide dismutases, catalase, and peroxidase by oxygen in *E. coli* K-12 and the protection afforded by these enzymes against the toxicity of oxygen and of streptonigrin form the substance of this report.

### MATERIALS AND METHODS

*E. coli* K-12 *his* *thr* (ATCC 23794) was grown at 37°C in a medium containing 3% Trypticase soy broth (Baltimore Biological Laboratories) and 0.5% yeast extract (Difco). Aerobic conditions were achieved by oscillation of 100-ml cultures in 500-ml Bellco culture flasks at 200 rpm on a rotary platform shaker. Static culture entailed growth in full flasks or test tubes without agitation. Anaerobiosis involved transfer of the medium directly from the autoclave into an anaerobic jar (Bioquest) followed, after a delay of 48 h, by inoculation and growth in an anaerobic glove box. Hydrogen gas and a palladium catalyst were used to scavenge oxygen from these anaerobic chambers.

Cells were harvested at 4°C by centrifugation for 15 min at  $10,000 \times g$  and were washed once with 0.1 M potassium phosphate (pH 7.0). Washed cells were suspended in 0.05 M potassium phosphate,  $10^{-4}$  M ethylenediaminetetraacetic acid (EDTA) at pH 7.8 and were disrupted for 3 min with a Branson W185 sonifier, operated at an output of 70 W. The cell suspension was kept at 4 to 6°C by working in an ice-

Handwritten notes: "H. P. Misra" and "N. Y. Fridovich" with a date "Nov 12 1976".

## Superoxide Dismutase: A Photochemical Augmentation Assay<sup>1</sup>

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Received November 29, 1976

Superoxide dismutases increase the rate of the aerobic photooxidation of dianisidine, sensitized by riboflavin. This rate enhancement appears to be due to the catalytic scavenging of  $O_2^-$ , which would otherwise nullify the overall photooxidation by reducing an intermediate oxidation state of the dianisidine. The effect of superoxide dismutase thus exposes a cyclical oxidation-reduction process, which would otherwise remain hidden from view. A sensitive and convenient augmentation assay for superoxide dismutase has been devised on the basis of this effect. It appears to be remarkably free of interferences and can be applied to crude soluble extracts of biological samples.

Superoxide dismutases are most frequently assayed by coupling a generator of  $O_2^-$  with an indicating scavenger for this radical. The enzyme then competes with the scavenger for the available  $O_2^-$  and inhibits the process being observed. The xanthine oxidase reaction, as a source of  $O_2^-$ , has thus been coupled with cytochrome c, as an indicating scavenger of  $O_2^-$  (1-4). Numerous variations on this theme, employing other sources of  $O_2^-$  and other indicating scavengers, have been devised (1, 5-9). Indeed, substances such as epinephrine (10), 6-hydroxydopamine (11), or pyrogallol (12) can act both as the source of  $O_2^-$  and as the indicating scavenger for this radical. In all of these cases, SOD<sup>2</sup> appears to inhibit and is measured on this basis.

Enzymes are usually assayed in terms of augmentation of reaction rates and attempts have been made to develop an augmentation assay for SOD. One such assay has been based upon an increase in the polarographic reduction wave for oxygen at a dropping mercury cathode (13). A spectrophotometric augmentation assay

for SOD would be desirable and one has been reported. It was based upon the ability of  $O_2^-$  to inhibit horseradish peroxidase and of SOD to prevent this inhibition (14). We now report a new spectrophotometric augmentation assay for SOD which is sensitive, reproducible, and applicable to crude extracts and which eliminates the need for extraneous enzymes, such as horseradish peroxidase.

### MATERIALS AND METHODS

Riboflavin was obtained from Eastman Organic Chemicals and dianisidine was from the Sigma Chemical Company. The copper-zinc-SOD was prepared from bovine erythrocytes (1), while the manganese-SOD (15) and the iron-SOD (16) were prepared from *Escherichia coli*. Illumination for the photochemical reactions was provided by a pair of parallel 20-W Sylvania Gro-Lux fluorescent tubes mounted on 6-in. centers in an aluminum foil-lined, open-ended box. Reaction mixtures in quartz cuvettes in cuvette holders at room temperature were placed midway between the fluorescent tubes and at intervals were transferred into a Gilford Model 2000 spectrophotometer for recording of absorbance. Dianisidine was dissolved in ethanol to 0.01 M and this ethanolic stock was added to reaction mixtures to give the desired final concentration. All other components were dissolved in 0.010 M potassium phosphate at pH 7.5.

### RESULTS

*The effect of SOD on the photooxidation of dianisidine.* Illumination of buffered aerobic mixtures of riboflavin and dianisi-

<sup>1</sup> This work was supported by Research Grants GM-10287 and HL-17603 from the National Institutes of Health, Bethesda, Maryland and by DAHC-0474-G-0194 from the United States Army Research Office, Research Triangle Park.

<sup>2</sup> Abbreviations used: SOD, superoxide dismutase; BeSOD, bovine erythrocyte superoxide dismutase.

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